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THE ROLE OF INTESTINAL BACTERIA IN ACUTE DIARRHEAL DISEASES

Annual Report

Sherwood L. Gorbach, M.D.

January 1980

Supported by

US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

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Tufts New England Medical Center
Boston, MA 02111

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The investigations discussed in this report covered five major areas:

1. Testing of E. coli isolated from humans (adults and infants) with diarrheal disease for production of enterotoxins and presence of colonization - specific surface antigens.

2. Isolation of bacterial pili which may function in binding of E. coli pathogens to the small bowel surface.

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b. chemical characteristics
c. preparation of pili-specific antisera
d. EM study of morphology
e. serologic characteristics of three pili serogroups.
f. pili cross linking with PDM
g. ^{3}H-NEM labelling of isolated pili
h. Binding of ^{3}H-NEM labelled pili to buccal mucosal cells.

3. Isolation of erythrocyte receptor involved in binding of piliated ETEC.

4. Adherence of ETEC to Eukaryotic cells

   a. buccal cell adherence assay
   b. scanning electron microscopy of adherence assay
   c. development of adherence assays using human intestinal cell lines.

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   b. plasmid analysis of spontaneous MR-HA^{-} derivatives of MR-HA^{+} ETEC strains.

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Publications during the past contract year.

Abstracts:

C.F. Deneke, G.M. Thorne, and S.L. Gorbach. Adhesive pili for enterotoxigenic Escherichia coli (ETEC) pathogenic for humans. International Conference Antimicrobial Agents and Chemotherapy. 1979.

C.F. Deneke, G.M. Thorne, and S.L. Gorbach. Adhesive pili for enterotoxigenic Escherichia coli pathogenic for humans. Fifteenth Joint Conference on Cholera. U.S. Japan Cooperative Medical Science Program, Washington, D.C., 1979.

Publications:

Thorne, G.M., Deneke, C.F., and S.L. Gorbach. Hemagglutination and adhesiveness of toxigenic Escherichia coli isolated from humans. Infect. Immun., 23: 690-699, 1979.

Deneke, C.F., Thorne, G.M., and S.L. Gorbach. Attachment pili from enterotoxigenic E. coli pathogenic for man. Infect. Immun. 26: 362-368, 1979.

Woytowicz, John M., Deneke, C.F., Thorne, G.M., and S.L. Gorbach. Glycophorin: an erythrocyte receptor for a class of pili found on enterotoxigenic Escherichia coli. Submitted to Infection and Immunity.

Deneke, C.F., Thorne, G.M., and S.L. Gorbach. Serogroups of attachment pili found on enterotoxigenic Escherichia coli of humans. Submitted to Infection and Immunity.

SECTION 1

Testing of E. coli isolated from humans with diarrheal disease for production of enterotoxins

During the Contract period, we have continued to screen Escherichia coli isolated from humans with diarrhea for their ability to produce LT and/or ST enterotoxins and for the presence of surface antigen(s) analogous to the K88 antigen of porcine enteropathic E. coli. An outline of the characteristics of known adherence surface antigens (pili) found on ETEC strains of human origin is given in Table 1.

The Evans group have described antigens CFA/I and CFA/II. Production of these two antigens always correlates with the appearance of fine filamentous projections covering the surface of the bacterial cell (pili) and mannose-resistant hemagglutination activity for specific rbc types. These two antigens have been found on ETEC of a limited number of O-groups, mostly in the O78 serogroup in the case of CFA/I. (These investigators have used an infant rabbit intestinal colonization assay as their adherence model).

Our studies which are detailed below provide evidence for at least 3 serologically distinct groups of surface antigens present on ETEC isolated from man. E. coli strains in each of the 3 serologic groups produce surface pili of "identical" size, morphology, and molecular weight. The presence of these pili shows no apparent correlation with the ability to cause MR-HA of a particular rbc type(s). In the past year our ETEC culture collection has doubled in size; E. coli strains belonging to various O-groups have been found to react with our 3 serologic types of pili-specific antisera (Tables 2-6). These data are described in detail in Section 2e.

SECTION 2

The studies described in the following Sections a-d have been published in Infection and Immunity.*

Pili: Adherence Antigens

a. Purification: Purification of adherence pili from ETEC by temperature-dependent red cell adsorption and elution was described previously (Annual Progress Report 1978). Pili isolated by this protocol have undergone further characterization.

b. Electron Microscopic (EM) Studies: Short, needle-like pili (5-10 nm x 300 nm) were found to be present on the surface of a number of different ETEC strains but not on non-enterotoxigenic control strains of E. coli (Fig. 1A to F). Pili preparations have also been examined by EM and appear to be homogenous. The isolated pili had the same diameter as the small pili on the intact bacterial cells (Figure 2).

c. Molecular Weight Determination: SDS-PAGE analysis of purified pili of various ETEC strains detected protein subunits of 12,500 and 13,100 molecular weight (Table 7).

d. Biological Reactivity: The isolated pili have also been shown to bind to human buccal cells under temperature conditions (37^{0} C) which prevent binding of these pili to erythrocytes.

The F_{ab} fragment of anti-pili immunoglobulin G antibody has been shown to block buccal cell binding by intact bacterial cells.

e. Serological Characterization: An important aim of this study has been to develop rabbit antisera against purified adherence pili for use in screening ETEC isolates from man. The results obtained with 84 ETEC are summarized in Table 8. We find that 53 of 84 ETEC strains (63%) react with one or more of the three pili-specific antisera. The ETEC were also

*Deneke, C.F., G.M. Thorne, and S.L. Gorbach, "Attachment Pili from Enterotoxigenic Escherichia coli Pathogenic for Humans." Infection and Immunity 1979, 26: 362-368.

examined for possible correlations between pili serogroup and O somatic antigens, enterotoxin profile, geographic origin, and/or MR-HA ability. The O somatic antigens of the ETEC strains are detailed in Table 9. In our study there is no apparent correlation of pili antigen type with specific somatic antigens. Thus, our findings are dissimilar to those of the Evans' group as indicated in Table 1. Likewise, there is no apparent correlation of pili serogroup with toxin profile (Table 10). ETEC strains which produce both toxins (LT/ST), LT-only, or ST-only have been found to synthesize each of the three pili types recognized by our antisera. In addition, there was no correlation of pili serogroup with MR-HA ability or geographic location of the strains (Tables 11,12).

We are currently preparing antisera against the surface pili present on ETEC strains 2016-10, $H326_c1$, CDC 5206-70, C921d, 54e-14 (Tables 2-4). These strains are MR-HA positive; yet do not react with the three pili antisera. One or more new pili serogroups may be recognized when these strains are studied further.

f. Pili Cross-Linking PDM: We attempted to clarify the antigenic interrelationships of the pili mediating MR-HA reactions and buccal cell adherence by Ouchterlony immunodiffusion analysis. Isolated pili are unstable, however, tending to disassociate into their monomeric units, which apparently have a single antigenic combining site. Behaving like a hapten, monovalent pili subunits proteins absorb anti-pili antibody and an immunoglobulin-mediated precipitation reaction does not occur. (The antibody titer against whole bacterial cells drops following pre-incubation with purified pili.). Therefore, we have cross-linked the pili

protein subunits with N, N'-p-phenylenedimaleimide (PDM). Using highly purified agarose (Seakem HGT-P) and barbitol buffer, we have been successful in performing immunodiffusion studies of the three pili serogroups recognized to date. The Ouchterlony analysis with PDM-linked pili antigens is shown in Figure 3. The precipitation lines show a reaction of non-identity (i.e. the precipitate lines intersect since the samples contain no antigenic determinant in common).

g. ^{3}H-NEM labeling of isolated pili: The isolated, partly purified pili have been labeled with ^{3}H-N-ethylmaleimide (NEM). The availability of isolated labeled pili will allow us to quantitatively study the attachment of these pili to various human cells, including buccal mucosal cells, red cell ghosts, and human small intestinal cells. Our preliminary experiments with ^{125}I labeling, using the lactoperoxidase glucose oxidase method, were unsuccessful due to very low incorporation of the label into the pili fraction and loss of buccal binding ability. Subsequently, we discovered that oxidants such as H_2O_2 denatured or inactivated the pili thereby making them unable to attach to buccal cells. We now have evidence from N-ethylmaleimide titrations that a single -SH group exists per pair of pili subunits (e.g. 12,500 and 13,100 mwt pair of subunits). This -SH group is apparently not involved with the binding reaction, since pili -mediated functions such as mannose-resistant hemagglutination occurs in the presence of Hg^{++} ions. The availability of these intact pili with the high specific activity of ^{3}H-NEM allows the study of the attachment of these pili to various cells.

h. Binding of ^{3}H-NEM labeled pili to buccal mucosal cells: Using the ^{3}H-NEM pili we have carried out an experiment to show that the labeled pili can be used in cell attachment studies. The time course of the attachment

of ^{3}H-NEM pili to human buccal cells is given in Figure 4 . The rate of attachment is not perfectly linear, decreasing slightly with time, and it is markedly slower than the attachment of the intact bacteria to buccal cells. This is a result of the large number of simultaneous attachment reactions occurring when the whole bacteria attach to buccal cells; that is, buccal binding by intact bacteria is the sum of multiple binding events, in contrast to the single binding event occurring with the labeled pili system. This reaction has not reached completion by 30 min. The supernatant after 5 min. incubation when added to fresh buccal cells shows the same level of binding. This finding suggests that after 5 min. of incubation the amount of functional ^{3}H-NEM-labeled pili has not significantly decreased.

In the future, this system will allow us to examine the pH and temperature effects on the binding of isolated pili to buccal cells and to compare the binding conditions for isolated pili and intact bacteria. We should be able to determine whether pili binding is the exclusive mechanism involved in the buccal cell attachment reaction. Kinetic studies, similar to those applied to enzymes, may allow us to further describe the interactions between the pili and eucaryotic receptors.

SECTION 3: Isolation of an erythrocyte receptor involved in binding of piliated ETEC strains.

Glycophorin, a major sialoglycoprotein on the erythrocyte membrane has been identified as a receptor for a class of pili found on enterotoxigenic E. coli. ETEC mediate mannose resistant hemagglutination (MR-HA) reactions presumably by adherence to red cells by their fur-like surface structures (pili). Red cell ghosts were also agglutinated by piliated ETEC strains (Table 13). However, trypsin treatment of the ghost preparations resulted in loss of MR-HA ability, except for bovine cells which retained reactivity. These data suggest that trypsin-treatment may remove a proteinaceous surface receptor, or that trypsin, in some other way alters the membrane surface.

To distinguish between the release of RBC surface receptors and other membrane alterations, the effect of trypsin-treated material on the MR-HA reaction of homologous RBC was tested. (Table 14). The addition of the trypsin-released material caused a qualitative decrease in the extent of the MR-HA reaction, implying that the receptor was removed by trypsin-cleavage and remained active. Trypsin-released material from human A, B, and O erythrocyte-ghosts reduced the MR-HA with three piliated ETEC strains, 193-4, 334, H10407; material released by trypsin from guinea pig erythrocyte-ghosts reduced the MR-HA with two strains, 193-4 and 334. Trypsin-released material from bovine erythrocyte-ghosts did not reduce the MR-HA with ETEC strains known to react with bovine erythrocytes.

Reduction of the MR-HA by the trypsin-released material was quantified using the micro-MR-HAI test (Table 15). Using guinea pig RBC and E. coli

strains 193-4 and 334, homologous and non-homologous trypsin-released materials dropped the titer as much as 16-fold, while the negative control strain 334LL did not hemagglutinate. The titer results were reproducible from day to day when fresh E. coli and guinea pig RBC suspensions were employed. Hemagglutination titers were also consistant when the microtiter trays were incubated at 37°C for 30 minutes (which reverses the MR-HA reaction) and reincubated at 0°C for 2 hours. Lysozyme did not reduce the titer of the MR-HA reaction when used in place of the trypsin-released material.

SDS electrophoretic analysis of intact glycophorin extracted by the standard lithium diiodosalicylate phenol method of Marchesi and Andrews* and the trypsin released material was performed. This analysis revealed the presence of identical molecular weight species that stained for both protein and carbohydrate. Hence, a glycoprotein (glycophorin) has been released from erythrocytes. This data suggests that glycophorin is an RBC receptor involved in the binding of piliated ETEC in the MR-HA reaction.

The importance of this work is not just that glycophorin is an RBC receptor but that we have isolated and purified it using methods that may be directly applicable to other human cell types. In the future, we plan to use similar methods to isolate pili receptors from buccal cells and from human small intestinal cells in order to elucidate the eucaryotic receptors for bacterial pili.

*Marchesi, V.T. and E.P. Andrews 1971 Glycoproteins: Isolation from cell membranes with lithium diiodosalicylate. Science 1974:1247-1248.

SECTION 4 Adherence of ETEC to Eukaryotic Cells

a. HUMAN BUCCAL CELL ADHERENCE ASSAY:

We have developed an adherence assay employing human buccal epithelial cells. (Thorne, G.M., Deneke, C.F. and Gorbach, S.L. Infection and Immunity 23:690-699, 1979). This system is attractive since it used human cells against human pathogens - the homologous system. Binding to human buccal epithelial cells was explored by us since it was known that another enteric pathogen, Vibrio cholerae, is present in high number in the oral cavity during acute and convalescent periods. Also, the buccal cells are easy to obtain, and they share certain similarities with the gastrointestinal mucosa. The buccal cells are part of a mucosal, secreting tissue contigusous with the gut, and ABO and Lewis blood group substances are known to be present in secretions and cells lining the entire GI tract.

We have employed two different systems to monitor the adherence of human toxigenic E. coli to the human epithelial cell suspensions: (1) staining the cell mixtures with an indirect fluorescent antibody technique; (2) radioactively labeled bacteria and a membrane filtration technique. These two binding assays have shown good agreement. These studies have allowed for the detection of E. coli strains with special surface properties which permit their binding to the buccal epithelial cells in the presence of mannose, a sugar which is known to inhibit cellular binding and HA reactions due to common type 1 pili of E. coli.

Of 32 toxigenic E. coli strains examined, 19 (59%) bound to the buccal cells, whereas none of the eight control E. coli strains did so (Mann-Whitney U test, $P = 0.007$). The control strains were non-toxigenic E. coli isolates from humans, enterotoxigenic E. coli isolates from animals, and E. coli K-12 containing the K88 or K99 plasmid; these strains

only exhibited background level adherence in this assay.

There was no correlation between ability to adhere to human buccal cells and mannose-resistant HA of bovine, guinea pig, human A and B erythrocytes. Similarly, the ETEC strains which react with 1 or more of the 3 pili antisera are variable in their MR-HA reactivity. (Table 16). Most strains which bind to buccal cells also react with pili antisera with the exception of the few strains in Group III. Other non-binding Group IV strains, control strains, and fecal isolates (Table 6) were unreactive in pili antisera.

b. SCANNING ELECTRON MICROSCOPY

The adherence of ETEC to human buccal cells was examined by scanning electron microscopy. Buccal cells alone, or following incubation with ETEC strains 334 or 334LL were applied to glass slides and fixed in 5% glutaraldehyde in PBS. The specimens were dehydrated in alcohol, critical point dried, fixed, mounted, and coated with gold. The scanning electron micrographs are shown in Figures 5-7 Individual bacilli can be seen attached to the buccal cells in the preparation containing ETEC strain 334. Buccal cells alone were free of attached organisms as shown in Figure 6 . Specimens prepared from mixtures of buccal cells and E. coli 334LL (enterotoxin negative, non-adherent MR-HA$^-$, plasmid free, derivative of ETEC strain 334) were found to be free of any attached organisms. In Figure 7, bacterial strands can be seen to interconnect the bacterial cells (ETEC 334) and to make contact with the buccal cell surface.

c. DEVELOPMENT OF ADHERENCE ASSAYS OF HUMAN ETEC STRAINS TO INTESTINAL CELL LINES

Because of our ongoing interest in the interactions of human bacterial pathogens with the small bowel of humans, we continue to search for an optimal eukaryotic intestinal cell or tissue. Studies have been performed

to utilize a tissue culture cell line of human origin as a uniform source of cells for an adherence assay. We have studied two normal fetal cell lines, FHA-74Int and MA-349. Each was highly trypsin- and calf serum-sensitive and failed to grow in the laboratory. Cell line HuTu-80, an adenocarcinoma of the duodenum, grew well but failed as a tool to allow discrimination between ETEC strains and non-toxigenic control strains. To date, we have been unsuccessful in our search for a human small intestine cell line that can be used in E. coli adherence studies.

In summary, our studies have identified three distinct pili or surface "sticky" antigens that are present on ETEC isolated from humans; While not a perfect system, the buccal cell assay does allow us to measure the affinity of various toxigenic E. coli fro human epithelial tissue. This should permit recognition of colonization ability in a particular strain, and therefore should separate E. coli truly pathogenic in humans from non-colonizing strains, both of which might still produce enterotoxin.

SECTION 5: Role of Plasmids in Adherence Antigen Production

In addition to examining ETEC of human origin for the presence of adherence ability and surface pili production, we have endeavored to study the genetic control of such characteristics.

a. Curing Experiments

Strain 334, originally isolated by the Principal-Investigator from a patient with diarrhea in Calcutta, was chosen for further study. This strain elaborates both ST and LT enterotoxins and causes mannose-resistant hemagglutination (MR-HA) reactions with a number of RBC types and produces our pili serogroup 1 surface antigen. Because of the possible plasmid nature of the adherence antigen , a number of plasmid "curing" procedures were used in attempts to isolate a MR-HA$^-$ derivative (Table 17). The usual curing agents, ethidium bromide and SDS, as well as growth at elevated temperatures, were not effective. Rifampicin and nalidixic acid treatment did yield MR-HA$^-$ derivatives. Rifampicin specifically interacts with RNA polymerase and has been shown to eliminate the F plasmid from E. coli. This antibiotic is also known to interfere with synthesis of plasmid-mediated surface components. The phenotypic characteristics and plasmid DNA bands exhibited by these derivative strains of 334 are detailed in Table 18. The phenotypic change exhibited by strain 334-27 (MR-HA negative under both test conditions)(Table 18)suggests that rifampin is affecting synthesis of both the adherence antigen and common fimbriae. Derivative 334P$^+$14 is also missing plasmid #4, yet it retains the MR-HA ability. The nalidixic acid resistant mutant (334 Nx^R-1, Table 18) was selected in order to perform plasmid superinfection matings. The absence of plasmids was noted after the strain was screened using a standard agarose electrophoresis technique. The nalidixic acid resistant derivative

of 334 was found to undergo additional plasmid loss following superinfection by a plasmid mediating tetracycline resistance which belongs to incompatibility group F_1. Derivative 334Nx^R-S1 which initially grew on selective media containing tetracycline (Tc) was found to revert to Tc^S when grown on antibiotic-free media. It has lost 5 of the 6 plasmid bands seen in 334 and does not appear to contain the Tc plasmid. The data given on Table 18 summarizes the information concerning toxin production, HA and MR-HA ability of strain 334 and its derivatives. From the phenotypic characteristics of derivatives 334 Nx^{R-1}, 334 P^+14, 334 Nx^R-SI, adherence antigen and toxin production are not related to the presence of plasmid bands #2, #3, or #4. It seems most likely that plasmid #1 (60 M daltons) will be found to determine both toxin and colonization antigen production. Plasmids mediating LT/ST production studied to date have been found to belong to a highly related group with a molecular size very similar to plasmid #1.

b. Plasmid Analysis of Spontaneous MR-HA^- Derivatives of MR-HA^+ ETEC Strains

Our laboratory has been examining ETEC of human origin for the presence of phenotype associated with adherence. In screening a large number of recently isolated strains we have noted a few strains that underwent simultaneous loss of MR-HA ability, buccal cell adherence, and agglutination with 334 pili antisera; upon further examination multiple plasmid loss was noted.

The adherence data of these ETEC strains and their MR-HA^- derivatives is given in Table 19. At present we are confronted by an array of data which strongly implicates plasmids in the control of production of these specialized surface antigens. Yet all attempts to demonstrate transfer of the incriminated plasmid have failed. During the past 6 months, efforts

have been made to insert transposable antibiotic resistance onto the "cryptic" plasmid suspected of involvement of pili production. Cloning experiments using restriction-ligation technology is also underway. These studies are detailed in the current Contract Renewal application.

TABLE 1

ETEC ADHERENCE ANTIGENS

TYPE:	ADHERENCE PILI	CFA/I	CFA/II
	Serogroup 1,2,3,		
MR-HA			
RBC Type	Human A, B$^{\pm}$	Human A^{+}	Bovine $^{+}$
	Bovine $^{\pm}$	Bovine $^{+}$	
	Guinea pig $^{\pm}$	Chicken $^{+}$	
MOLECULAR Weight:	13,00	23,800	
	12,500		
SIZE:	5-10nm	7nm	
TISSUE:	Human buccal	Rabbit	Rabbit
	Mucosa	Intestine	Intestine
ENTEROTOXIN(s)	LT/ST	LT/ST	LT/ST
	ST^{+}	ST^{+}	
O-Group:	06, 08, 015	06, 08, 015	06, 09
	025, 027, 078	025, 078	085
	0128, 0148, 0149		

TABLE 2

ESCHERICHIA COLI STRAINS EXAMINED

Human Strains	Origin	Serotype	Enterotoxins Status	MR-HA[2] AB	GP	BOV	Pili Serogroups[3]
334	AD India	O15:H11	LT/ST	+	+	+	1
334LL	LP	O15:H11	-	-	-	-	NR
193-4	AD India	N.T.	LT/ST	+	+	+	1
H10407	AD India	O78:H11	LT/ST	+	-	-	1,3
H10407P	LP	O78:H11	LT^+	-	-	-	NR
Tx-1	ID Texas	O78:K80:H12	ST	+	-	+	2,3
Tx-85	ID Texas	O78:k80:H12	ST	+	-	+	2,3
B2C	AD Viet Nam		LT/ST	-	-	-	2
B7A	Ad Viet Nam		LT/ST	-	-	-	NR
214-4	AD MD		ST	-	-	-	NR
K108c3	AD Kenya	-	LT/ST	-	-	-	
K324c1	AD Kenya	O8:O60:H9	LT/ST	-	+	-	2
K344c2	AD Kenya	-	LT	-	-	-	NR
K130c1	AD Kenya	-	LT/ST	-	-	-	NT
K135c2	AD Kenya	-	-	-	-	-	2
K325c3	AD Kenya	-	-	-	-	-	2
K326c5	AD Kenya	O25:h42	LT/ST	+	-	-	2
K328c4	AD Kenya	-	-	-	-	-	NT
K325c1	AD Kenya	-	LT	-	-	-	NT
TD462c1	AD Mexico	O6:H16	LT/ST	-	-	-	2,3
TD260c1	AD Mexico	O6:H16	LT	-	-	-	2
TD514c1	AD Mexico		-	-	-	-	NT
TD412c1	AD Mexico		LT/ST	-	-	-	NT
TD514c2	AD Mexico		-	-	-	-	NT

TABLE 2 CONTINUED

Human Strains	Origin[1]	Serotype	Enterotoxins Status	MR-HA[2] AB	GP	BOV	Pili Serogroups[3]
TD427c2	AD Mexico		LT	-	-	-	NT
TD213c2	AD Mexico	0128	ST	+	-	-	?
TD234c4	AD Mexico		LT	-	-	-	NT
TD219c1	AD Mexico	06:H16	ST	+	-	-	3
TD451c2	AD Mexico		LT/ST	-	-	-	NT
TD327c2	AD Mexico	05	-	-	-	+	2
M9800-5	USA(Crater Lake)	06:K 15:H16	ST	-	-	+	2
SS34560	USA(Crater Lake)	06:H16	ST	-	-	-	2
SS34561	USA(Crater Lake)	06:H16	ST	-	-	+	2
D370855	Dacca	?	LT/ST	+	-	+	2,3 neg
D444	Dacca	?	LT/ST	-	-	+	2
D563	Dacca	?	LT/ST	+	-	+	3
D513	Dacca	?	LT/ST	-	-	-	2
D542	Dacca	?	LT/ST	+	-	+	3
D481	Dacca	?	LT/ST	+	-	+	3
D280551	Dacca	?	LT/ST	+	-	-	2
D370844	Dacca	?	LT/ST	+	-	+	2,3 neg
D524	Dacca	?	LT/ST	+	-	+	2
D280561	Dacca	?	LT/ST	-	+	-	2,3 neg
TD235CH$_4$	AD Mexico	?	LT	-	-	-	NR
TD425C2	AD Mexico	?	LT	-	-	-	NR
M403C3	Morocco		ST	-	-	-	
M409C1	Morocco		ST	-	-	-	

TABLE 2 CONTINUED

1. AD - adult diarrhea

   LP - lab passage

   ID - infant diarrhea

2. Mannose-resistant hemagglutination of washed human A, B, guinea pig and bovine RBC's at $4^{o}C$.

3. NR - non-reactive in antisera

   NT - not typable due to auto-agglutination.

TABLE 3

ETEC ISOLATES PROVIDED BY DR. I. KAYE WACHSMUTH

of THE CDC. ATLANTA, GA.

THE ORIGIN OF THESE STRAINS IS DETAILED IN TABLE

STRAINS	ORIGIN	SEROTYPE	TOXINS	AB	MR-HA GP	BOV	Pili SEROGROUPS
CDC-69-2707	Maryland	015: H11	LT/ST	+	+	-	1
CDC-70-5206	New Mexico	0128: H21	ST	+	+	-	NR
CDC-70-5610	Dacca	015: H11	LT				
CDC-70-5726	Pakistan	078: H11	LT	-	-	-	NR
CDC-70-5203	New Mexico	0128: H21	ST	+	+	-	1
CDC-70-5727	Pakistan	078: H11	LT	-	-	-	1
CDC-70-5605	Dacca	020: H -	LT/ST				
CDC-70-5729	Dacca	015: H11	LT	-	-	-	1
CDC-72-5467	Washington, D.C.	06: H16	ST	-	-	-	2
CDC-72-5460	Washington, D.C.	06: H16	LT/ST	-	-	-	2
CDC-73-1119	California	078: K80: H12	LT/ST	-	-	-	
CDC-73-0562	?	027: H20	ST	-	-	-	NR
CDC-77-1781	?	025: K98: H -	LT	-	-	-	NR
CDC-77-1780	Mexico	025: K98: H -	LT	-	-	-	NR
CDC-69-5400	?	015: H11	ST	+	+	+	1,3
CDC-70-0423	Vietnam	0148: H28	LT/ST	-	-	-	
CDC-71-1694	Foreign	027: H20	ST	-	+	-	2
CDC-72-5448	?	0148: H28	LT/ST	-	-	-	2
CDC-72-1782	Foreign	027: H20	ST	-	-	-	2
CDC-72-1943	?	027: H20	$ST^+LT?$	-	-	-	
CDC-72-2513	Massachusetts	06: H16	ST^+LT^+	-	-	-	2,1
CDC-73-0563	?	027: H20	ST^+	-	-	-	2

TABLE 3

STRAINS	ORIGIN	SEROTYPE	TOXINS	MR-HA AB	MR-HA GP	MR-HA BOV	Pili Serogroups
CDC-73-2694	?	0149: H7	ST^+	-	-	-	1?
CDC-76-1086	Arizona	015: H -	LT/ST	-	-	-	
CDC-76-0100	?	078: K80: H12	(ST^+) *	-	-	-	
CDC-77-2520	Louisiana	0128: H7	(ST^+) *	-	-	-	1
CDC-77-2521	Louisiana	015: H -	LT/ST	-	-	-	1
CDC-77-3502	?	015: H11	LT/ST	+	+	+	1
CDC-77-2368	North Dakota	0128: H7	(ST^+) *	-	-	-	?
CDC-77-1782	Cruise Ship	0148: H28	ST/LT	-	-	-	2
CDC-77-2417	Massachusetts	015: H11	ST/LT	-	-	-	1,3
CDC-79-1203	Cruise Ship	025: H -	LT	-	-	-	

1. NR - non-reactive in antisera

* K.W. - reported these cultures ST negative upon retesting

TABLE 4

ETEC ISOLATES FROM CASES OF HUMAN DIARRHEAL

IN ETHIOPIA AND SWEDEN

(STRAINS PROVIDED BY DR. TORKEL WADSTROM, UPPSALA SWEDEN)

STRAIN	ORIGIN	SEROTYPE	ENTEROTOXINS	HUMAN AB,	GUINEA PIG,	BOVINE	PILI SEROGROUP
C922f	Sweden	06: K15:M	LT/ST	-		-	2
C921d	Sweden	06: K15:M	LT/ST	-	-	-	2
96-8	Ethiopia	078:KN:NM	LT/T	+		-	NT[b]
E80	Ethiopia	06:K15:M	LT/ST	+		+	2
2016-10	Ethiopia	ND:M	ST$^+$	+	-	-	NT
2016-16[a]	Ethiopia	ND:M	-	-	-	-	NT
1628-15	Ethiopia	078:KN:NM	LT/ST	+		+	3
E271a	Ethiopia	06:K15:H$^-$	LT/ST	-		-	2
5?e-14	Ethiopia	078:KN:NM	LTST	+		-	NT

[a] Strain 2016-16 is an enterotoxin negative, MR-HA$^-$, spontaneous derivative of strain 2016-10.

[b] NT = non-typable, strains spontaneously agglutinate.

TABLE 5

ETEC ISOLATES FROM CASES OF HUMAN DIARRHEAL DISEASE

IN HONDURAS

(PROVIDED BY DR. R. B. SACK, BALTIMORE, M.D.)

STRAIN	-TOXINS-	MR-HA AB	MR-HA GP	MR-HA BOV	Pili Serogroup
H 326 $_{c1}$	LT/ST	+	-	+	
H 410 $_{c1}$	LT/ST	+	-	+	
H 415 $_{c1}$	LT	-	-	-	
H 150 C6B	LT	-	-	-	1,3
H 109 C6I	LT	-	-	-	
H 426 $_{c2}$	LT	-	-	-	
H 556 $_{c11}$	LT	-	-	-	
H 142 C5A	ST	-	-	-	
H 439 $_{c3}$	LT	-	-	-	
H 326 $_{c2}$	LT	-	-	-	3
H 111 C6A	LT	-	-	-	
H 341 $_{c5}$	LT	-	-	-	2
H 218 $_{c5}$	ST	-	-	-	2
H 326 $_{c3}$	ST	+	-	+	3
H 449 $_{c3}$	ST	-	-	-	

TABLE 6

E. COLI CONTROL STRAINS

Animal Origin	Serotype	MR-HA[1] AB	GP	BOV	Pili Serogroups
RDEC-1 (Ovine diarrhea)	?	-	-	-	NR
P307 (Porcine diarrhea)	-K88^{+}	-	+	-	NR
LAB DERIVED					
K 12					NT
K 12 K88	K88	-	+	-	NT
K 12 K99	K99	-	-	-	NT
ADULT FECES (Non-TOXIGENIC)					
H10405 (LT ? Klipstein)	?	-	-	-	NR
HS	?	-	-	-	NR
CD-1	?	-	-	-	NR
Ec #1	?	-	-	-	NR
#7	?	-	-	-	NR
#8	?	-	-	-	NR
#9	?	-	-	-	NR
#10	?	-	-	-	NR
#11	?	+			NR
#12	?	-	-		NR
#13	?	+			NR
#14	?	-	-		NR
#15	?	-	-		NR
#16	?	-	-		NR

TABLE 6 CONTINUED

Animal Origin	Serotype	MR-HA[1] AB	GP	BOV	Pili Serogroups[2]
ADULT FECES (cont'd)					
#17	?	+			NR
#18	?	-	-		NR
#19	?	-	-		NR
#20	?	+			NR
#21	?	-	-		NR

1. Mannose-resistant hemagglutination of washed human A, B, guinea pig and bovine RBC's at $4^{o}C$.

2. NR - non-reactive in antisera

   NT - not typable due to auto-agglutination.

TABLE 7

DECTECTION OF K-LIKE PILI ON STRAINS OF ENTEROTOXIGENIC E. COLI ISOLATED FROM MAN

Strains	Serotype	Enterotoxins	Buccal Cell Adherence	Mannose-Resistant[2] Hemagglutination	Pili Detected BY SDS-PAGE[3]	Electron Microscopy[4] 5-10 nm	Electron Microscopy[4] 13-20nm
334	015:H11	LT/ST	+	+	+	+	+
193-4	N.T.[1]	LT/ST	+	+	+	+	+
TX-1	078:K80:H12	ST	+	+	+	+	+
D542	N.T.	LT/ST	+	+	+	+	+
D481	N.T.	LT/ST	+	+	+	+	+
D563	N.T.	LT/ST	+	+	+	+	+
CONTROL NONTOXIGENIC STRAINS							
334LL	015:H11	-	-	-	?	-	-
CD-1	N.T.	-	-	-	?	-	-
K12 K88	N.T.	-	-	+	+	+	-

[1] N.T. not typeable

[2] Mannose-resistant hemagglutination reactions were performed using human A & B and guinea pig red blood cells at 0°C.

[3] SDS-PAGE - Proteins of 12,500 and 13,100 molecular weight detected by SDS-PAGE after RBC attachment and elution procedure.

[4] These larger structures (13 to 20 nm) morphologically resemble flagella.

TABLE 8

REACTION OF 84 ETEC STRAINS WITH PILI SPECIFIC ANTISERA

PILI SEROGROUPS	NUMBER OF STRAINS	%
Group 1	12	14.3
Group 2	24	28.6
Group 3	7	8.3
Group 1,3	4	4.8
Group 2,3	6	7.1
Total Pili Reactive	53	63.1
Total Negative	31	36.9
Total	84	100.0

TABLE 9

O GROUPS OF ETEC STRAINS WITH ADHERENCE PILI

Pili Serogroup	Number of Strains	O Group 06,08	O Group 015	Other
1	12	0	7	078,0128,0149,NT
2	24	7	0	025,027,0128,0148,NT
3	7	1	0	0128,NT
1,3	4	1	1	078
2,3	6	1	0	078,NT
Negative	31	0	2	020,025,027,078,0128, 0148,NT

TABLE 10

ENTEROTOXIN PROFILES OF ETEC STRAINS

	ENTEROTOXINS		
PILI SEROGROUPS	LT/ST	LT	ST
Group 1	6	2	4
Group 2	12	4	8
Group 3	3	1	3
Group 1/3	2	1	1
Group 2/3	4	0	2
Negative	8	15	8
TOTALS	35 (41.7%)	23 (27.4%)	26 (30.9%)

TABLE II

ETEC TESTED FOR MR-HA WITH RED CELLS

FROM: HUMAN A, B, BOVINE, GUINEA PIG

	MR-HA	
	Postive	Negative
Pili Serogroup 1	6	6
2	8	16
3	6	1
1,3	2	2
2,3	4	2
Unreactive	5	28
	31	53

TABLE 12

Geographic Origin of ETEC Isolates Examined for Presence of 3 Serologic Pili Types

Country	Pili Serological Groups					(NT,NR)
	Type 1	Type 2	Type 3	Type 1,3	Type 2,3	negative
U.S.A.	5	2		1	2	4
"Cruise Ships"		1				1
Mexico		2	1		1	3
Honduras		2	2	1		8
Sweden		2				0
Morocco						2
Kenya		4				1
Ethiopia		2	1			4
India	2			1		
Pakistan	1					1
Bangladesh	2	4	3		3	
Vietnam		1				2
"Foreign"		2				
Unknown	2	2		1		5
TOTALS	12	24	7	4	6	31

TABLE 13. The MR-HA Reaction Of ETEC And Intact Erythrocytes, Erythrocyte-Ghosts, and Trypsin-Treated Ghosts.

ETEC Strain	Erythrocyte Blood Type [a]	Intact Erythrocytes [b]	Erythrocyte-Ghosts [b]	Trypsin-Treated Ghosts [b]
193-4	A	++	++	-
	B	+	+	-
	O	-	-	-
	GP	++	++	-
	BV	+	+	-
334	A	+	±	-
	B	+	±	-
	O	-	-	-
	GP	+	±	-
	BV	++	±	±
H10407	A	+	±	-
	B	++	±	-
	O	+	+	-
	GP	-	-	-
	BV	-	-	-
K324c1	A	-	-	-
	B	-	-	-
	O	-	-	-
	GP	-	-	-
	BV	+	±	±

[a] Human A,B,O; guinea pig, GP; and bovine, BV, red blood cells

[b] Red blood cell-bacterial aggregation: ++, clumpy; +, grainy; ±, slightly grainy; -, no aggregation.

TABLE 14. The Effect of Trypsin-Released Material on The MR-HA Reaction.

	ERYTHROCYTE BLOOD TYPE [a]				
ETEC Strain	A	B	O	GP	BV
193-4	D[b]	NC	-	D	D
334	D	NC	-	D	D
H10407	NC	D	D	-	-
K324c1	-	-	-	-	D

[a] Erythrocyte call type, A - human A; B - human B; O - human O; GP - guinea pig; BV - Bovine

[b] NC , no change ; D, decrease; I, increase; - , negative.

TABLE 15 . The MR-HAI Reaction Between ETEC, Intact Erythrocytes And Trypsin-Released Materials.

ETEC Strain	Inhibitor [a]	Titer [b]
193-4	-	512
	GP	128
	A	64
	B	32
	lysozyme	512
334	-	512
	A	32
	B	32
334LL	-	0
	GP	0
	A	0
	B	0

b - 1% or 3% guinea pig red blood cells were used as the indicator red blood cells.

a - Inhibitor concentrations: GP, guinea pig - 180 µg/ml
A, human A - 400 µg/ml
B, human B - 380 µg/ml
lysozyme - 100 µg/ml

TABLE 16. Comparison of Agglutination Reaction and/or Buccal Cell Adherence of Human Toxigenic E. coli strains

Strain	Serotype[a]	MR-HA[b] AB	MR-HA[b] GP	MR-HA[b] BOV	Buccal Adherence[c]	Pili Serogroup[d]
Group I						
334	015:H11	+	+	+	246 ± 48 (11)	1
193-4	NT	+	+	+	171 ± 30 (3)	1
TX-85	078:K80:H12	+	-	+	101 ± 10 (4)	2,3 CFA/I$^+$
TX-1	078:K80:H12	+	-	+	147 ± 28 (4)	2,3 CFA/I$^+$
K324c1	08:06:H9	-	+	-	172 ± 66 (3)	2
H10407	078:H11	+	-	-	253 ± 73 (4)	1,3 CFA/I$^+$
K326c5	025:H42	+	-	-	136 ± 9 (2)	2
D542	NT	+	-	+	115 ± 11 (3)	3
D563	NT	+	-	+	102 ± 37 (3)	3
D481	NT	+	-	+	135 ± 37 (3)	3
D280551	NT	+	-	-	128 ± 20 (4)	2
M9800-5	06:K15:H16	-	-	+		2 CFA/II
Group II						
H10407P	078:H11	-	-	-	255 ± 104 (7)	NR
K325c3	-	-	-	-	132 ± 55 (4)	2
K135c2	-	-	-	-	470 ± 56 (3)	2
TD235c4	-	-	-	-	144 ± 39 (5)	NR
TD462c1	06:H16	-	-	-	275 ± 86 (3)	2,3
214-4	NT	-	-	-	108 ± 14 (5)	NR
B2C	06:H16	-	-	-	183 ± 40 (4)	2
D444	NT	-	-	-	112 ± 21 (4)	2
Group III						
TD213c2	0128	+	-	-	46 ± 6 (7)	?
TD219c1	06:H16	+	-	-	50 ± 28 (3)	3
D370855	NT	+	-	+	50 ± 10 (6)	2,3
D370844	NT	+	-	+	39 ± 5 (4)	2,3
TD327c2	05	-	+	+	30 ± 20 (2)	?
D280561		-	+	-	86 ± 14 (4)	2,3
D524		+	-	+	17 ± 9 (3)	2
Group IV						
B7A	0148:H28	-	-	-	75 ± 58 (4)	NR
M403c3		-	-	-	28 ± 11 (7)	NR
K344c2		-	-	-	84 ± 42 (5)	NR
TD451c2		-	-	-	76 ± 15 (4)	NT
D513		-	-	-	22 ± 15 (4)	2
M409c1		-	-	-	25 ± 5 (4)	?

TABLE 16 CONTINUED

Strains	Serotype[a]	MR-HA[b] AB	MR-HA[b] GP	MR-HA[b] BOV	Buccal Adherence[c]	Pili Serogroup[d]
Control, nontoxigenic						
334LL	O15:H1]	-	-	-	13 ± 3(10)	NR
CD-1		-	-	-	37 ± 9 (5)	NR
HS		-	-	-	61 ± 17 (5)	?
RDEC-1		-	-	-	43 ± 14 (4)	NR
P307		-	+	-	38 ± 29 (3)	NR
K-12		-	-	-	31 ± 15 (4)	NT
K-12 K88		-	+	-	81 ± 38 (4)	NT
K-12 K99		-	-	-	48 ± 19 (4)	NT

---

[a]NT - Nontypable

[b]Mannose-resistant hemagglutination (MR-HA) of washed human group A(A), group B(B), or guinea pig(GP) erythrocytes at 0°C.

[c]Counts of number of bacteria/buccal cell ± standard error of the mean. The number of assays is shown in parentheses.

[d]Agglutination of whole bacteria by pili-specific antisera: serogroup 1, produced against 334 pili, serogroup 2, produced against M9800-5 pili, serogroup 3 produced against D563 pili. NR = non-reactive in seros, NT = non-typable due to autoagglutination. CFA/I,II are indicated for strains reported by the Evans group to produce these antigens.

TABLE 17 SUMMARY OF CURING EXPERIMENTS

USING E. COLI 334 (LT/ST, HA^+)

"CURING" TREATMENT	NO. COLONIES TESTED	NO. COLONIES HA-
1. GROWTH AT 42°C	62	0
2. GROWTH AT 44°C	105	0
3. GROWTH AT 48°C	56	0
4. GROWTH AT 49°C	14	0
5. ETHIDIUM BROMIDE		
30 ug/ml	110	0
60 ug/ml	57	0
90 ug/ml	3	0
120 ug/ml	1	0
240 ug/ml	2	0
6. LAURYL SULFATE, SODIUM SALT		
1%	62	0
2%	100	0
3%	58	0
4%	55	0
5%	50	0
7. RIFAMPIN		
40 ug/ml	43	0
20 ug/ml	27	0
2 ug/ml	60	18
0.5 ug/ml	26	1
8. ACRIDINE ORANGE		
5 ug/ml	10	0
9. NEOMYCIN SULFATE		
5 ug/ml	9	0
10. NALIDIXIC ACID		
50 ug/ml	1	1

TABLE 18 BIOLOGICAL PROPERTIES AND PLASMID COMPONENTS OF E. COLI 334 AND ITS DERIVATIVE STRAINS

STRAIN	METHOD OF ISOLATION	HA TESTS[1]		ST[2] TOXIN	LT[3] TOXIN	PLASMIDS[4] LARGE		SMALL			
		0°C (+) mannose (K-like antigen)	24°C (-) mannose (type 1 pili)			#1	#2	#3	#4	#5	#6
334		+	+	+	+	+	+	+	+	+	+
334 NX^R-1	nalidixic acid	+	+	+	+	+	-	-	+	+	+
334-27	rifampin	-	-	+	+	+	+	+	-	+	+
334P+15	rifampin	+	-	+	+	+	-	+	-	+	+
334LL	storage	-	+	-	-	-	-	-	-	-	-

Guinea pig erythrocytes were used for the hemaglutination (HA) tests

1. HA test (+) mannose, at 0°C is specific for K88-like sticky antigen.
   HA test (-) mannose, at 24°C is an indication of the presence of type 1 pili.
2. ST toxin was assayed in the suckling mice
3. LT toxin was assayed in YI adrenal cell cultures
4. Plasmid DNA components were observed following agarose gel electrophoresis as described.
   - #1 is the largest plasmid seen at the top of the gel, #6 is the smallest and fastest moving species seen at bottom of the gel.

TABLE 19

BUCCAL ADHERENCE
OF MR-HA$^+$ AND MR-HA$^-$ ISOGENIC STRAINS

STRAIN	MEAN NO. BACTERIA ± SEM / BUCCAL CELL	
	MR-HA$^+$	MR-HA$^-$
334	246 ± 48	13 ± 3
$M403_{C3}$	192 ± 98	19 ± 10
$K324_{C1}$	172 ± 66	38 ± 4

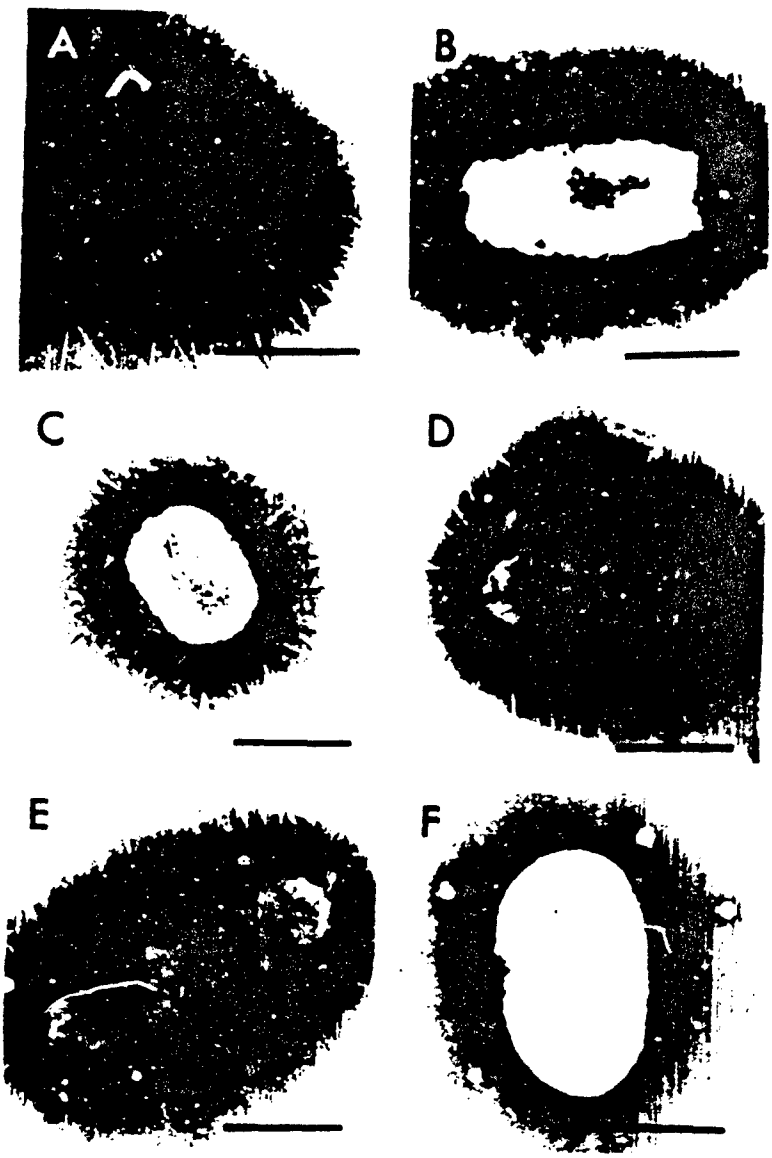

Figure 1. Five strains of toxigenic E. coli and one non-enterotoxigenic strain negatively stained with 2% phosphotungstic acid. A. 334; B. 193-4; C. D563; D. D481; E. TX-1; F. 334LL (negative control). Attachment pili are small,5-10 nm, needle-like structures. Bar = 1.0μ.

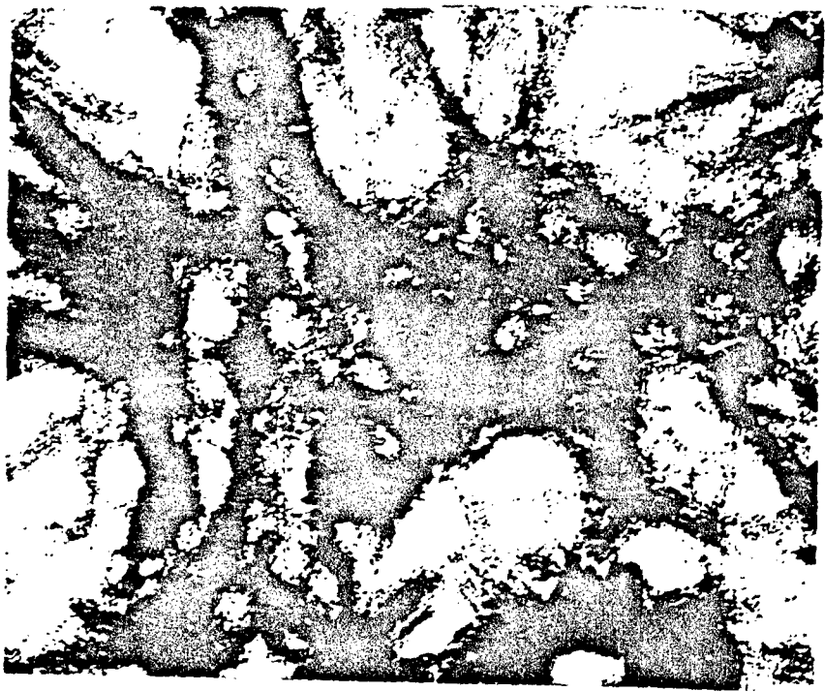

Figure 2. Pili preparation of ETEC strain 193-4 stained with 4% uranyl acetate. Bar = 1.0μ.

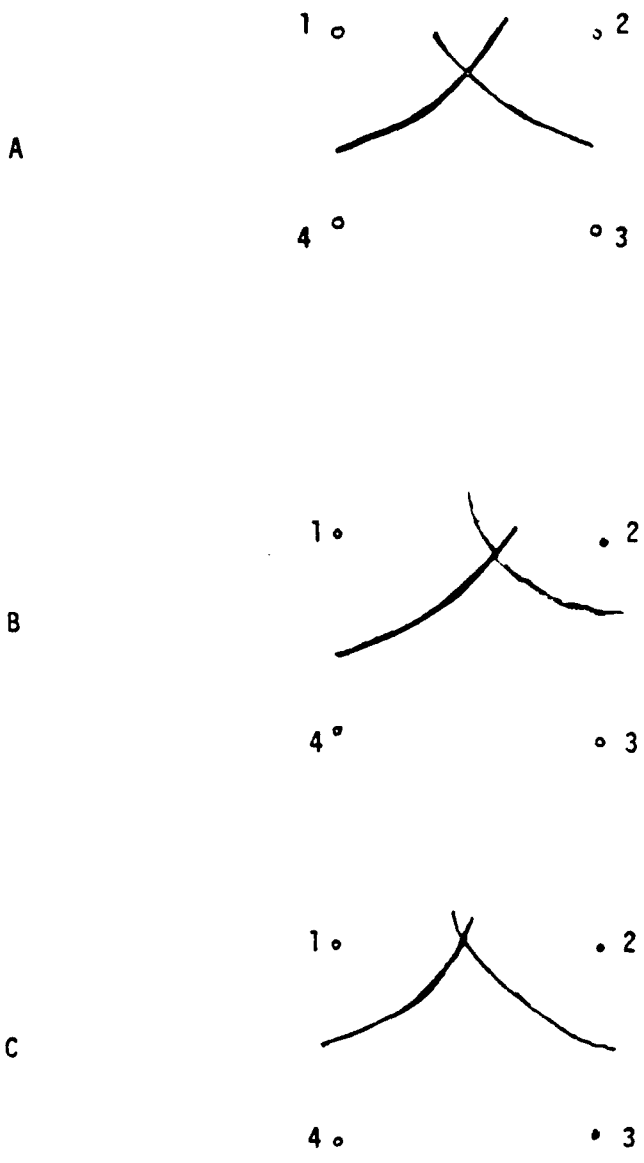

Figure 3.

Ouchterlony Immunodiffusion Analysis of 3 Pili Antigens.

Figure 3.

Immunodiffusion showing antigenic non-cross reactivity of three pili antigens.

A. Well 1, 334 pili antisera; Well 2, D563 pili antisera; Well 3, 334 pili cross linked with N,N'-p-phenylenedimaleimide (PDM): Well 4, D563 pili cross linked with PDM.

B. Well 1, 334 pili antisera; Well 2, M9800-5 pili antisera; Well 3, 334 pili cross linked with PDM; Well 4, M9800-5 pili cross linked with PDM.

C. Well 1, D563 pili antisera; Well 2 M9800-5 pili antisera; Well 3, D563 pili cross linked with pDM; Well 4, M9800-5 pili cross linked with PDM.

Figure 4. Attachment of ^{3}H-N-ethylmaleimide labeled pili to human buccal mucosal cells. Pili were labeled by reaction with ^{3}H-N-ethylmaleimide for 5 minutes, then the free label separated from the protein bound fraction by gel filtration using BioGel P2. The isolated, labeled pili were mixed with buccal cells in 1% mannose-PBS. At appropriate times aliquots were centrifuged in a microfuge for 30 seconds and washed twice. The final wash supernatant was also counted to insure complete removal of unbound label. This supernatant only contained background levels of radioactivity.

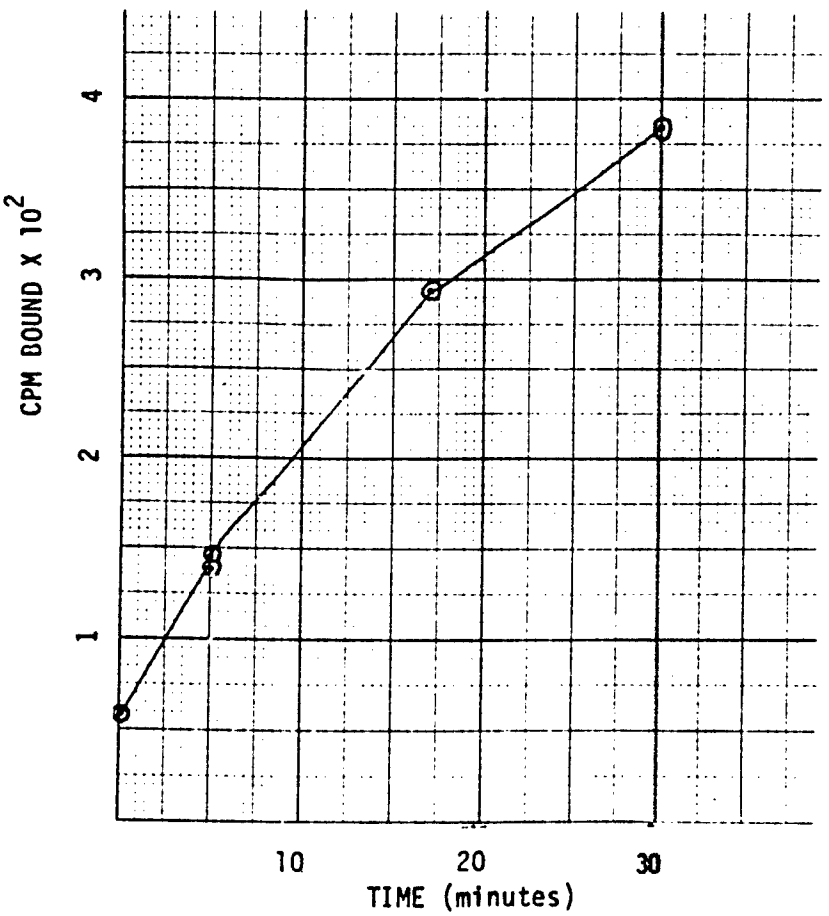

Figure 5. Scanning electron micrograph of human buccal cells with attached ETEC strain 334 (x 1500)

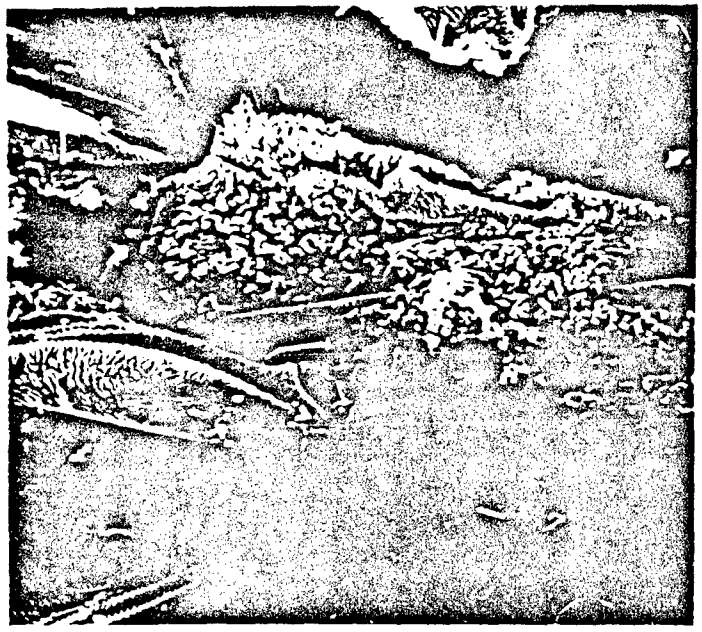

Figure 6. Scanning electron micrograph of human buccal cells with 334LL (toxin$^-$, MR-HA$^-$ derivative of ETEC strain 334). (x 1500)

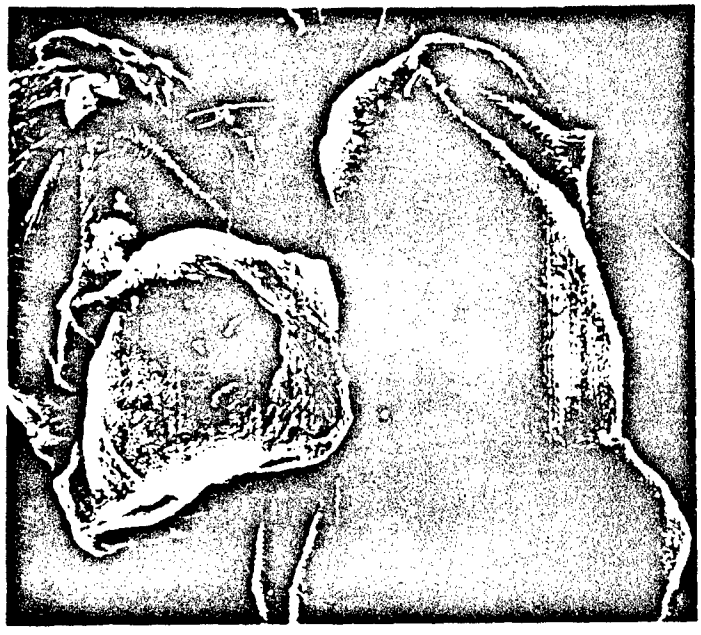

Figure 7. Scanning electron micrograph of human buccal cells with attached ETEC 334 (x 20,000)